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A micrometabolic inhibition test for the estimation of poliovirus neutralizing antibodies *

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A modified micrometabolic inhibition test for the titration of poliovirus neutralizing antibodies is described. The effects of varying both the duration and the temperature of incubation of the serum–virus mixtures were examined. Overnight incubation resulted in a 2–3-fold increase in the titre of the hyperimmune sera tested. The method is simple and economical and appears to be suitable for antibody surveys.

Since 1954, when a metabolic inhibition test for the estimation of poliovirus neutralizing antibodies was first introduced by Salk et al. (1954) a number of modifications of the standard technique have been published (Melnick & Opton, 1956; Shand, 1961; Mussett et al., 1961).

The micromethod described here, adapted from that used by Stott & Tyrrell (1968) in their rhinovirus studies, has been used in this laboratory for more than 4 years. It has proved satisfactory for the estimation of neutralizing antibodies to both poliovirus and coxsackievirus group B in the sera of patients (Grist & Bell, 1970; Bell & Grist, 1970).

Attempts to increase the sensitivity of the test without adversely affecting its reproducibility have been made by varying the duration and temperature of incubation of the serum–virus mixtures. The results of these tests are presented here.

METHODS

Viruses

Prototype strains of poliovirus type 1 Brunenders, type 2 MEF1, and type 3 Saukett were adapted to growth in HeLa cell cultures.

Antisera and human sera

Two groups of monospecific antisera prepared in monkeys to each of the 3 types of poliovirus were used. One group was obtained from the Research Reference Reagents Branch, National Institutes of Health, Bethesda, Md., USA (referred to as NIH antisera in the text); the other, designated British Standard Poliomyelitis Antisera, was obtained from Dr F. T. Perkins, National Institute for Medical Research, Hampstead Laboratories, London.

Sera from 10 children aged 3 years in a nursery school in Glasgow were available for study.

All antisera and sera were inactivated at 56°C for 30 min before use.

Cells

HeLa cells were grown in Eagle's MEM containing 5% fetal calf serum, 0.1% sodium hydrogen carbonate, penicillin (100 IU/ml) and streptomycin (100 IU/ml). Subcultures were prepared each week; the chelating agent "Versene" was used and the bottles were reseeded at 8×10^4 cells/ml.

Media

The media in the metabolic inhibition test were modified from those described by Shand (1961). Both contained phenol red indicator:

A. Diluent for viruses and sera	
Parker's medium 199	86 ml
fetal calf serum	5 ml
5.6% $NaHCO_3$ solution	3 ml
2M $MgCl_2$ solution	1 ml
10% glucose solution	3 ml
penicillin	100 IU/ml
streptomycin	100 IU/ml
B. Diluent for cells	
Eagle's MEM	86 ml
glutamine	1 ml
fetal calf serum	5 ml
5.6% $NaHCO_3$ solution	2 ml
2M $MgCl_2$ solution	1 ml
10% glucose solution	3 ml
antibiotics as in medium A	

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Table 1. Results of a typical test [a]

A. Poliovirus type 1–NIH antiserum titration [a]

Serum titre									Serum control at 80
80	160	320	640	1 280	2 560	5 120	10 240	20 480	
0	0	0	0	0	+	+	+	+	0
0	0	0	0	0	+	+	+	+	0
0	0	0	0	+	+	+	+	+	0
0	0	0	0	+	+	+	+	+	0

[a] Poliovirus type 1 used to 100 TCD_{50} (i.e., diluted 10^{-4}).

B. Poliovirus type 1 titration

Dilution							Cell control 100 %
10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	
+	+	+	+	+	0	0	0
+	+	+	+	0	0	0	0
+	+	+	+	0	0	0	0
+	+	+	+	+	0	0	0

C. Cell titration

Concentration					
100 %	0	0	0	0	0
50 %	—	—	—	—	—
25 %	+	+	+	+	+
12.5 %	+	+	+	+	+

[a] 0 = yellow; — = orange; and + = red. The colour readings show the titre of poliovirus 1 to be 10^6 TCD_{50} per 0.025 ml, and the 50 % titre of the serum to be 1 280.

Micrometabolic inhibition test procedure

Rigid, disposable, flat-bottomed, plastic microtitre plates (Bio-Cult, IS/FB/96) were used together with microtitre loops and droppers delivering unit volumes of 0.025 ml. Serial dilutions of antisera or children's sera were made in medium A. Approximately 100 TCD_{50} of virus in 0.025 ml was added to each serum dilution and the mixtures were incubated for different periods and at different temperatures. Then 15 000 HeLa cells in 0.1 ml of medium B were added and the plates were sealed with transparent adhesive tape and incubated at 37°C for 3 days. Included routinely in each test were (1) serum toxicity controls consisting of serum at the lowest dilution used in the test, (2) a titration of cell suspension to ensure that the correct concentration of cells was used in the test, and (3) a virus titration to confirm that the virus was used at 100 TCD_{50}. At the end of 3 days' incubation, the sealing tape was removed and the plates, loosely covered by sterile lids or aluminium foil, were held at 37°C for 2 hours. By this time the carbon dioxide produced by metabolism of the cells had escaped and the medium in the cups had changed in colour from yellow or orange to red. The plates were then resealed with tape and incubated for a further 2 days at 37°C before the test was read. The results of a typical test are shown in Table 1. A yellow colour indicated virus neutralization or normal cell growth; red indicated virus growth or cell toxicity. All serum and virus titrations were calculated by the method of Kärber (1931) with the use of 50% infective or protective endpoint readings, respectively.

RESULTS

Virus titrations

Before the titration of poliovirus neutralizing antibodies was begun, the three types of poliovirus were first titrated before the addition of HeLa cells; 4 cups per dilution and three different incubation periods were used—1½ h at room temperature, 3 h at room temperature, and finally 3 h at room temperature followed by overnight incubation at 4°C. Each titration was carried out in duplicate and repeated on four separate days using freshly prepared virus dilutions, cells, and media to test the reproducibility of the results. The observed titres of the three polioviruses are listed in Table 2. The reproducibility of the results appeared adequate. Overnight incubation of viruses before addition of the cells did not result in a significant drop in titre.

Antiserum titrations

In the titration of the NIH antisera 4 cups were used per dilution. The serum–virus mixtures were incubated as described above. The tests were repeated on 8 different days with freshly prepared antiserum and virus dilutions, cells, and media. The reproducibility was good since none of the 9 groups showed more than 2-fold variation in average titre: 5 of the 72 readings deviated 2-fold from the mean. Overnight incubation of serum–virus mixtures resulted in an approximately 3-fold increase for all 3 poliovirus types when compared with incubation for 1½ h at room temperature. Thus the former method was more sensitive for the detection of poliovirus neutralizing antibodies in these hyperimmune sera. On the basis of this observation the British Standard Poliomyelitis Antisera were titrated twice in parallel with the NIH antisera, only these two incubation times being used. Again a 2–3-fold increase in titre for all 3 poliovirus types was observed.

Table 2. Poliovirus titrations [a] on four different days

Virus	Day	Incubation time		
		1½h room temperature	3 h room temperature	3 h room temperature +4°C overnight
poliovirus 1	1	6.50	6.50	6.50
	2	6.75	6.50	6.50
	3	6.25	6.25	6.25
	4	6.25	6.25	6.25
	gmt [b]	6.44	6.38	6.38
poliovirus 2	1	6.50	6.50	6.50
	2	6.25	6.50	6.75
	3	6.75	6.75	6.50
	4	6.50	6.75	6.50
	gmt	6.50	6.62	6.56
poliovirus 3	1	6.50	6.50	6.50
	2	6.25	6.50	6.50
	3	6.75	6.75	6.75
	4	6.50	6.75	6.75
	gmt	6.50	6.62	6.62

[a] Titres expressed as dex (see Haldane, 1960).
[b] gmt = geometric mean titre.

Children's sera

Ten sera from 3-year-old children in a day nursery were available for study. These children had given negative, low-titre or high-titre positive results in previous tests with the short incubation period. The 10 sera were titrated in parallel with the British Standard Antisera and the results are shown in Table 3. The increases in titre observed after overnight incubation of serum–virus mixtures were more variable than those that had been seen with the hyperimmune sera. In only two tests (children no. 2 and 3) did " negative " results (titre <8) by the short method convert to " positive " results by the overnight incubation method.

DISCUSSION

The advantages of the present micrometabolic inhibition technique over the more commonly used cell culture methods in tubes are numerous. Economy of serum required for testing is paramount, particularly for large-scale virus surveys of the community. The use of easily maintained HeLa cells and commercially available microtitre apparatus makes it possible for laboratories not otherwise equipped to perform general virus investigations to undertake such poliovirus studies. Moreover the test permits the screening of several hundred sera per week with minimal incubation and refrigeration space. Since the readings are scored by colour and not by observation of cytopathic effects, no microscopes are required.

Many workers using metabolic inhibition techniques have found difficulty in interpreting the results because of intermediate colour changes between yellow and red. Many have also used liquid paraffin for sealing their cultures. This test overcomes both of these problems. Uncovering the plates for 2 h at 37°C and thus allowing the carbon dioxide to escape before resealing avoids most of the intermediate colour changes. The use of a sealing film that requires no prior sterilization is also an advantage.

No significant reduction in virus titres of any of the 3 poliovirus types was seen after overnight incubation at 4°C before the addition of cells. Titration of hyperimmune sera by this method resulted in a

Table 3. Antibody titres of human sera with different incubation times

Serum no.	Polio 1		Polio 2		Polio 3	
	1½ h room temperature	3 h room temperature +4°C overnight	1½ h room temperature	3 h room temperature +4°C overnight	1½ h room temperature	3 h room temperature +4°C overnight
1	<8	<8	<8	<8	<8	<8
2	<8	<8	<8	<8	<8	12
3	<8	<8	8	12	<8	24
4	12	24	12	64	24	64
5	8	32	48	96	<8	<8
6	24	64	48	96	16	32
7	128	256	128	256	8	32
8	384	512	128	1 024	64	256
9	1 024	1 024	24	64	8	64
10	128	192	512	4 096	2 048	2 048
British standard antisera	240	640	160	480	640	1 280

3-fold increase in titre, with no effect on the reproducibility of the test. In no instance was a lower serum titre observed after overnight incubation.

Not unexpectedly the results obtained with the children's sera were more variable. Where an increase in titre was detected, this averaged from 2- to 4-fold, the effect of overnight incubation being greater in the sera with the lowest titres, i.e., in the range most significant for evaluation of the immune status of a population. However, the number of sera tested was too small for the results to be conclusive.

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RÉSUMÉ

MICROTEST D'INHIBITION MÉTABOLIQUE POUR LE TITRAGE DES ANTICORPS NEUTRALISANTS ANTIPOLIOMYÉLITIQUES

Les auteurs décrivent une variante du test d'inhibition métabolique destiné au titrage des anticorps neutralisants antipoliomyélitiques. Dans cette épreuve, on met en présence le virus et l'immunsérum, puis on ajoute au mélange une suspension de cellules HeLa et du rouge de phénol comme indicateur. En cas de neutralisation du virus, la croissance cellulaire se poursuit et le milieu vire au jaune. Si le virus n'est pas neutralisé, les cellules

sont détruites et l'indicateur se maintient au rouge.

Des essais ont été faits à l'aide d'immunsérums préparés chez le singe et de sérums humains. Ils ont montré qu'en incubant le mélange sérum-virus pendant une nuit à 4°C, avant d'introduire les cellules, on obtenait des titres d'anticorps 2 à 3 fois plus élevés sans nuire à la reproductibilité du test.

Les auteurs insistent sur les avantages de la méthode, notamment: économie de sérum, facilité d'obtention des cultures cellulaires et lecture aisée des résultats.

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